emc has a role in dorsal appendage fate formation in Drosophila oogenesis

Mechanisms of Development 122 (2005) 961–974

www.elsevier.com/locate/modo

emc has a role in dorsal appendage fate formation in *Drosophila* oogenesis

Sofia Papadia[a], George Tzolovsky[a], Debiao Zhao[b], Kevin Leaper[a], Dorothy Clyde[c], Paul Taylor[d], Eva Asscher[e], Graeme Kirk[a], Mary Bownes[a,*]

[a]*Institute of Cell and Molecular Biology, University of Edinburgh, Edinburgh EH9 3JR, UK*
[b]*Roslin Institute, Roslin, Midlothian EH25 9PS, UK*
[c]*Biosciences, University of Kent, Canterbury CT2 7NJ, UK*
[d]*MRC Human Reproductive Sciences Unit, University of Edinburgh Centre for Reproductive Biology, Edinburgh EH16 4SB, UK*
[e]*Wellcome/CR UK Gurdon Institute and Department of Anatomy, Cambridge University, Cambridge CB2 1QR, UK*

Received 23 December 2004; received in revised form 26 April 2005; accepted 5 May 2005
Available online 31 May 2005

Abstract

extramacrochaetae (*emc*) functions during many developmental processes in *Drosophila*, such as sensory organ formation, sex determination, wing vein differentiation, regulation of eye photoreceptor differentiation, cell proliferation and development of the Malpighian tubules, trachea and muscles in the embryo. It encodes a Helix-Loop-Helix transcription factor that negatively regulates bHLH proteins. We show here that *emc* mRNA and protein are present throughout oogenesis in a dynamic expression pattern and that *emc* is involved in the regulation of chorionic appendage formation during late oogenesis. Expression of sense and antisense *emc* constructs as well as *emc* follicle cell clones leads to eggs with shorter, thicker dorsal appendages that are closer together at base than in the wild type. We demonstrate that *emc* lies downstream of *fs(1)K10*, *gurken* and *EGFR* in the Grk/EGFR signalling pathway and that it participates in controlling Broad-Complex expression at late stages of oogenesis.

Keywords: Oogenesis; Extramacrochaetae; Grk/EGFR pathway; Cell fate; Dorsal appendage formation; Br-C

1. Introduction

The gene *extramacrochaetae* (*emc*) encodes a transcription factor and is involved in many diverse processes at various stages of *Drosophila* development (Campuzano, 2001 for a review). It negatively regulates the *achaete-scute* Complex (AS-C) (Botas et al., 1982) and encodes a Helix-Loop-Helix (HLH) protein (Ellis et al., 1990; Garrell and Modolell, 1990). Emc belongs to class V of HLH proteins (Massari and Murre, 2000), together with the Id proteins (Inhibitor of differentiation) in mammals (Benezra et al., 1990). Proteins of this class lack the basic region thus are unable to bind DNA (Davis et al., 1990) and are negative regulators of class I and class II HLH proteins by forming inactive heterodimers with them.

Emc regulates Sensory Organ (bristle) formation in a concentration-dependent manner by forming heterodimers with Daughterless and the AS-C proteins (Van Doren et al., 1991; Cabrera et al., 1994). It, therefore, limits the amount of Daughterless (Da) and Scute (Sc) available to form active heterodimers that promote Sensory Mother Organ (SMC, the precursor cell of SOs) formation. Thus, only cells with sufficiently high levels of Da, Ac and Sc to titrate Emc and to activate the downstream genes of the neural differentiation pathway are able to become SMCs (Campuzano, 2001). Emc function is also required in early embryogenesis for sex determination. The interaction between X-linked 'numerator' bHLH proteins (encoded by *sisterless-α*, *scute* and *sis-c*) and autosomal 'denominators' such as Emc, Daughterless and Deadpan regulates the X:A ratio which, in turn, determines whether *Sex-lethal* will be transcribed or not, thus regulating sexual fate (Younger-Shepherd et al., 1992). Only in females, with twice the concentration of Sc compared to males, are there sufficient active Sc/Da

* Corresponding author. Tel.: +44 131 650 5369; fax: +44 131 650 5371.
E-mail address: mary.bownes@ed.ac.uk (M. Bownes).

0925-4773/$ - see front matter © 2005 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.mod.2005.05.002

heterodimers to overcome the inhibitory effects of Emc and other putative autosomal-linked negative regulators and to activate Sxl (Campuzano, 2001). Later in embryogenesis *emc* is required for a number of processes, including Malpighian tubule formation, tracheal and muscle development and visceral mesoderm migration, by interacting with EGFR, Achaete, Breathless and Trachealess, Da and Twist (Cubas et al., 1994; Ellis, 1994). In the developing eye Emc and Hairy negatively regulate the expression of *atonal*, a bHLH gene which together with Da is necessary for the specification of R8, the first photoreceptor in an ommatidium. Downregulation of *emc* and *hairy* at specific locations by the Notch signalling pathway is essential for the initiation of eye development (Baonza and Freeman, 2001). In the wing Emc is involved in vein differentiation (de Celis, 1998, review). *emc* is expressed in the intervein cells flanking the veins where *Notch* and *E(spl)mβ* are expressed (Baonza et al., 2000). Recently Adam and Montell (2004), in a screen for genes involved in cell fate decisions in the ovary, showed that Emc has a role in follicle cell differentiation by inducing or maintaining EYA (Eyes Absent, a negative regulator of polar/stalk cell fate) in the epithelial follicle cells upon signalling from Notch, during early oogenesis.

The *Drosophila* ovary consists of about 16 ovarioles which are independent egg assembly lines. Oogenesis starts in the germarium, where a germline stem cell divides asymmetrically to give a daughter stem cell and a differentiated cystoblast, which undergoes four mitotic divisions with incomplete cytokinesis (in germarium region 1). This results in the formation of a 16-cell germline cyst within which the cystocytes (germline cells) are interconnected by cytoplasmic bridges called ring canals. The germline cyst becomes enveloped by follicle cells as it moves through germarium region 2, while within the cyst one of the two pro-oocytes (cystocytes with four ring canals) differentiates into the oocyte and the other germline cells become nurse cells (King, 1970; Spradling, 1993).

The Grk/EGFR signalling pathway establishes and regulates axis polarity (Neuman-Silberberg and Schüpbach, 1993; Queenan et al., 1997). The oocyte nucleus moves from the posterior to the anterior of the oocyte during stages 7–8 of oogenesis and as a result *gurken* (*grk*) mRNA and protein are expressed at the dorsal anterior region of the oocyte from stage 9. Gurken signals to the Epidermal Growth Factor Receptor (EGFR) on the surface of the adjacent follicle cells and through an elaborate regulation involving several genes that include *rhomboid, pointed, argos, spitz, kekkon-1, mirror* and *Broad-Complex* (Br-C), EGFR signalling results in two subsets of dorsal anterior follicle cells either side of the dorsal midline to become determined to migrate anteriorly and secrete the dorsal appendages (Ruohola-Baker et al., 1993; Morimoto et al., 1996; Deng and Bownes, 1997; Deng and Bownes, 1998; Wassermann and Freeman, 1998; Van Buskirk and Schüpbach, 1999; Zhao and Bownes, 1999; Cooperstock and Lipshitz, 2001). Downregulation of EGFR signalling results in a reduction of the number of cells exhibiting a dorsal fate leading to a reduction in the distance between the dorsal appendages whereas EGFR overexpression results in dorsalised eggs and embryos by increasing the distance between dorsal appendages (Neuman-Silberberg and Schüpbach, 1993, 1994; Queenan et al., 1997). In this paper, we show that *emc* contributes to the determination of dorsal follicle cell fate and that it acts downstream of Grk/EGFR and upstream of Br-C.

2. Results

2.1. The dynamic expression pattern of extramacrochaetae in oogenesis

A *lacZ* enhancer-trap fly line *261/31* was chosen for analysis based on its β-galactosidase expression in subsets of follicle cells during oogenesis. This line was provided from Peter Deak's collection (Deak et al., 1997). β-galactosidase expression in *261/31* was detected throughout *Drosophila* oogenesis with particularly strong expression from stages 2 to 11. Initially, staining is detected in all follicle cells (stages 2–6—Fig. 1B_1). At stages 7–8 follicle cells over the oocyte are stained more intensely (Fig. 1B_1) and at stage 9 *lacZ* is highly expressed in a band of follicle cells at the boundary between the oocyte and the nurse cells and in the migrating follicle cells that cover the anterior two thirds of the oocyte (results not shown), as well as in the stretched follicle cells that remain over the nurse cells. During stages 10–11 *lacZ* expression is localised to the centripetal follicle cells, the nurse cell-associated follicle cells and the dorsal anterior follicle cells; in the latter, at stage 10A expression in the columnar follicle cells becomes restricted to the region over the dorsal anterior of the oocyte. This expression pattern remains until stage 11 when two distinct subsets of stained follicle cells are defined (Fig. 1$B_{1,2}$). As the dorsal–ventral axis is established during this stage, the dorsal anterior expression of this gene suggested a potential role in dorsal–ventral patterning of the egg. At stage 12 expression is localised in two discrete groups of dorsal anterior follicle cells on either side of the dorsal midline (Fig. 1B_3), which move anteriorly over the forming dorsal appendages as the oocyte develops (stage 13) but are now connected as one band covering the dorsal midline (Fig. 1B_4).

A fragment of genomic DNA isolated following plasmid rescue of *261/31* corresponded to the known gene *extramacrochaetae* (*emc*) (Fig. 1A). Full length cDNA was obtained from Garrell and Modolell (1990) and used in all subsequent experiments. The expression pattern of *emc* during oogenesis was determined by mRNA in situ hybridisation to whole mount ovaries (Fig. 1C_{1-6}). Expression of *emc* is detected in all somatic follicle cells from stage 1 (in region 3 of the germarium) until stage 6

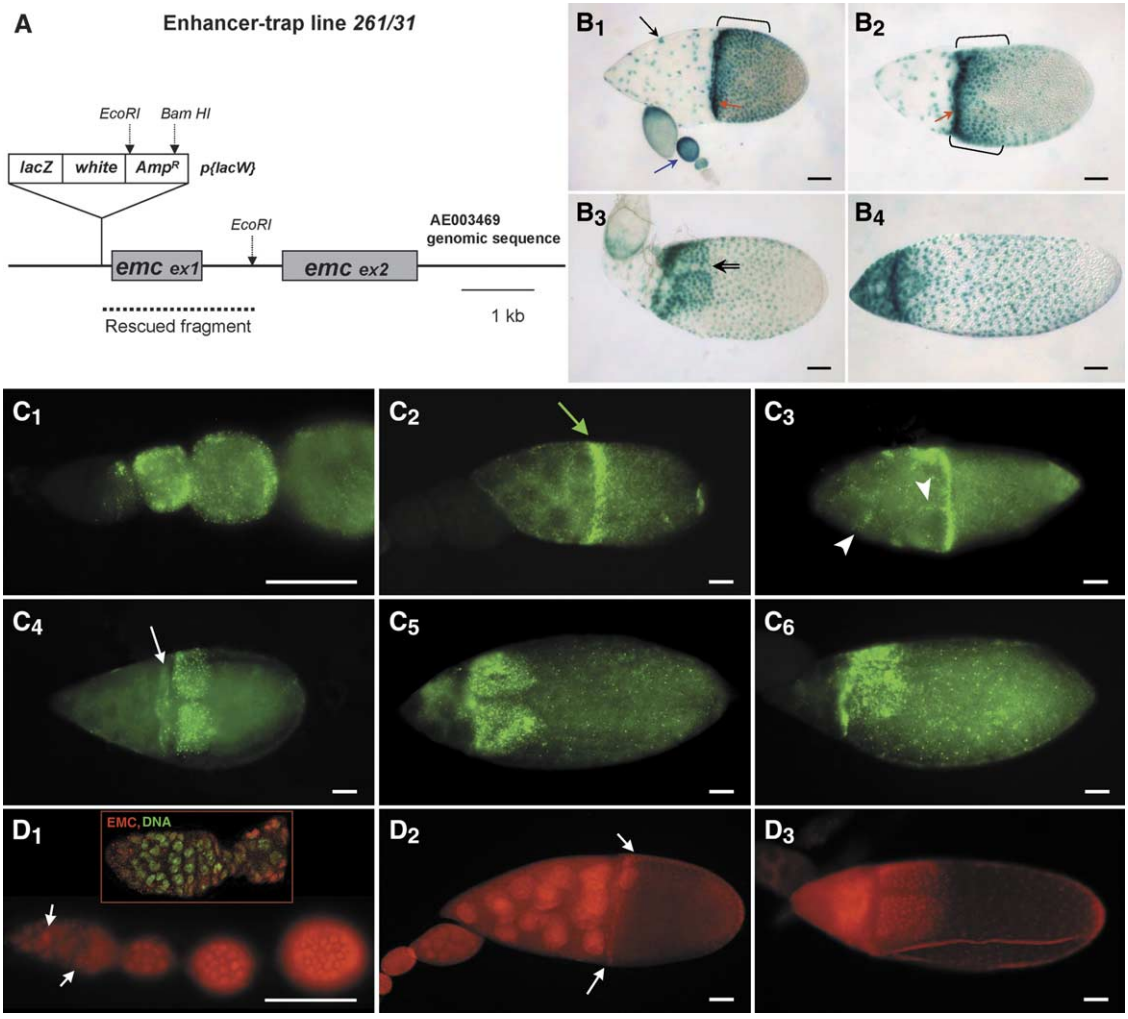

Fig. 1. (A) A genomic map of *261/31* showing the site of *P{lacW}* insertion 72 bp 5′ from exon 1 of *emc*. Exons are shaded grey and labelled ex1 and ex2 for exons 1 and 2, respectively. The solid line represents genomic DNA and the dotted line represents the rescued DNA fragment. ($B_{1–4}$) β-galactosidase staining pattern in egg chambers of the enhancer-trap line *261/31*. For details, see text. (B_1) An ovariole containing the germarium (not stained), egg chambers in early stages of oogenesis (stages 3, 6 and 8 are presented here) and a stage 10A egg chamber (main picture). Blue arrow, *lacZ* expression in all follicle cells covering the germline cells in the early stages of oogenesis; black arrow, expression in the stretched follicle cells covering the nurse cells; red arrow, expression in the centripetal cells and, bracket, in the area over the dorsal anterior of the oocyte. (B_2) Stage 11 egg chamber with *lacZ* expression localised in the centripetal cells (red arrow) and in two groups of lateral dorsal anterior follicle cells (brackets). (B_3) In a stage 12 egg chamber, the two groups of dorsal anterior follicle cells are separated by a gap on the dorsal midline (double black arrow). Expression is also retained in a ring of follicle cells at the border between the oocyte and nurse cells. (B_4) Strong *lacZ* expression in follicle cells over the forming dorsal appendages and in a small area over the dorsal midline (stage 13). B_1 is a lateral view while ($B_{2,4}$) are top views (dorsal is at the top) and in (B_3) dorsal is marked by the direction of the arrow. ($C_{1–6}$). In situ hybridisation analysis in *OrR* ovaries using a DIG labelled *emc* RNA probe and ($D_{1–3}$) Emc antibody staining in *OrR* ovaries. (C_1) *emc* mRNA (green) is expressed ubiquitously in stages 1 (germarium region 3)–6 of oogenesis, in the follicle cells and the nurse cell cytoplasm. Occasionally expression was also observed in region 2 of the germarium (not shown). ($C_{2–3}$) At stage 10A (C_2) *emc* expression is very strong in a band of follicle cells that cover the anterior of the oocyte (green arrow). There is a low level of expression in the nurse cell cytoplasm that continues in later stages. The stretched follicle cells over the nurse cells also express *emc* (arrowheads in C_3—stage 10B egg chamber). (C_4) By stage 11 *emc* is expressed in the centripetal cells (arrow) and in two discrete groups of lateral dorsal anterior follicle cells either side of the dorsal midline. (C_5) Towards stage 12 these two groups of follicle cells become restricted to dorsal anterior positions on either side of a thin gap along the dorsal midline and (C_6) later in stages 12–13 there is a continuous area of *emc* expression covering the dorsal anterior of the developing oocyte, including the dorsal midline. (D_1) Emc protein (red) is expressed in the germarium (arrows) and in the follicle cells surrounding early stage egg chambers. Inset, magnified germarium with Emc (red) and Sytox Green (green, DNA stain). Emc expression in follicle cells. (D_2) The nurse cell and oocyte nuclei strongly express Emc. In the follicle cells Emc expression is stronger in the centripetal and dorsal anterior cells (arrows). (D_3). At stage 12 Emc is expressed in two dorsal anterior patches of follicle cells and the nurse cell nuclei. (C_{4-5}) are dorsal views, (C_6) and (D_3) are dorso-lateral and (D_2) is a lateral view. Scale bar = 50 μm. Inset in (D_1) is magnified 1.2×. In all figures anterior is to the left.

(Fig. 1C_1 and not shown). Subsequently *emc* expression becomes restricted to a tight band of follicle cells at the nurse cell-oocyte boundary and to the stretched follicle cells over the nurse cells at stage 10A and 10B (Fig. 1$C_{2–3}$). During stage 11 expression is refined to two subsets of follicle cells either side of the dorsal midline (Fig. 1$C_{4–5}$). Expression persists in these follicle cells as they migrate anteriorly, secreting the dorsal appendages, but *emc* is now

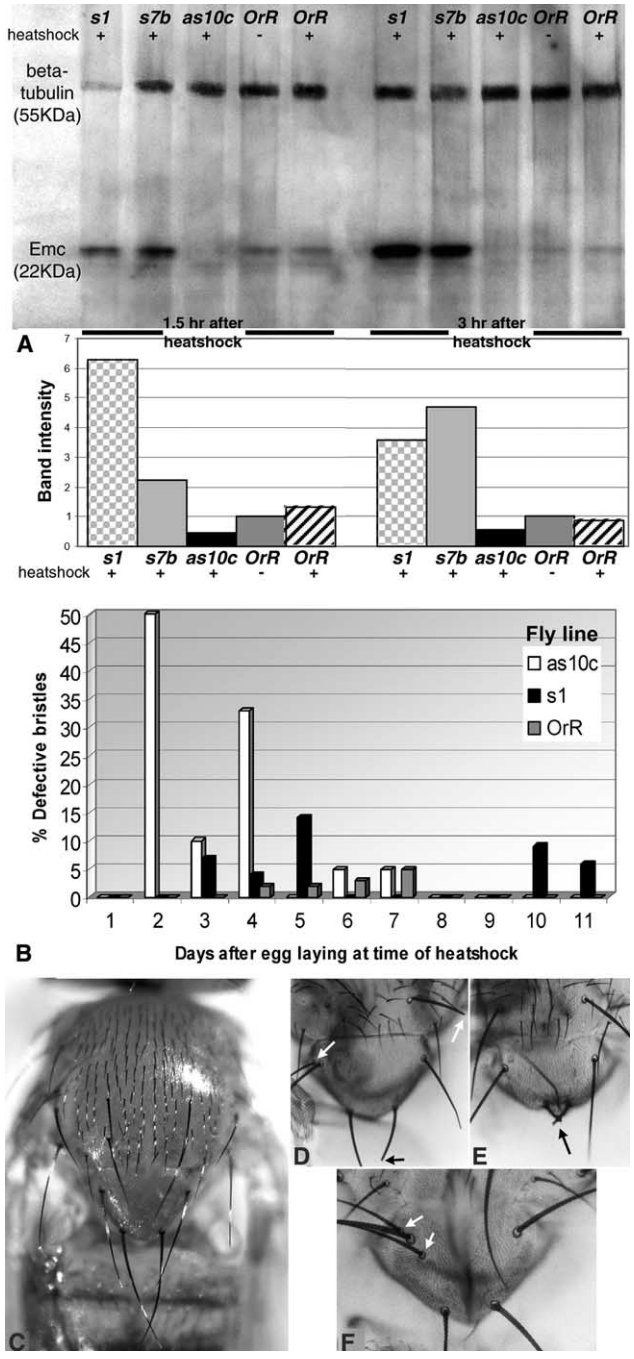

Fig. 2. (A) Western blot with Emc antibody and anti-β-Tubulin (loading control) to ovaries from wild-type, *emc* sense (*s1, s7b*) and antisense (*as10c*) fly lines. In lanes 1–5 the ovaries were dissected 1.5 h after heatshock and in lanes 6–10 the ovaries were dissected 3 h after heatshock. The low endogenous level of Emc in wild-type flies is not affected significantly by the heatshock treatment. Emc protein expression is significantly increased in heatshocked *s1* and *s7b* flies (lanes 1 and 2) and stays at high levels with time (lanes 6 and 7). In contrast, no Emc protein is detected in the heatshocked *emc* antisense line as10c, neither 1.5 nor 3 h after heatshock (lanes 3 and 8). The graph shows the relative intensity of bands after normalising for β-Tubulin [(Emc intensity − background)/(β-tubulin intensity − background)]. All values are expressed relative to the non-heatshocked *OrR* sample (negative control; value = 1). (B) Graph representing the phenotypic effect of the heatshock relative to the age of flies at the time of heatshock. The number of flies with defective bristles, including truncated bristles and extra bristles (but not missing bristles), which hatched following heatshock at various developmental stages is shown for one antisense (*as10c*—white), one sense (*s1*—black) and a wild type (*OrR*—grey) line. (C) Wild-type bristle pattern (*OrR*). (D) One extra scutellar bristle and truncated thickened bristles. (E) Short abnormal scutellar bristles. (F) Two additional scutellar bristles. Abnormal bristles are marked with arrows.

expressed in a single larger area covering the dorsal midline (Fig. 1C$_6$). By stage 14 expression is limited to a small group of follicle cells located between the dorsal appendages (data not shown).

Emc protein follows the mRNA expression pattern and is detected by immunostaining with an *emc* antibody in follicle cells of all stages, starting from the germarium (Fig. 1D$_1$ and inset). Protein is not expressed very strongly at stages 7–8. It is present in all germline cell nuclei in mid- and late oogenesis (stages 8–13) (Fig. 1D$_2$), in the centripetal cells at stage 10 and in the posterior polar cells (stage 10–11). At stages 11 and 12 Emc is expressed in two dorsal anterior subsets of follicle cells either side of the dorsal midline (Fig. 1D$_3$), during stage 12 these dorsal follicle cells migrate anteriorly and at stage 13 Emc is only detected in the follicle cells of the developing dorsal appendages (data not shown and Fig. 3A).

2.2. Generation of transgenic emc lines

To investigate further the role of *emc* in oogenesis we generated transgenic fly lines expressing sense and antisense RNA (Deng et al., 1999). Full-length *emc* cDNA in either the sense (s) or antisense (as) orientation was expressed under a heatshock (hs) promoter. From the eight heatshock-sense-*emc* (*hs-s-emc*) lines and 12 heatshock-antisense-*emc* (*hs-as-emc*) lines established, lines *s1*, *s7b* and *as10c* were selected for further study since they showed the highest egg abnormality rates when heatshocked (refer to Fig. 5A,B).

Western blot analysis was used to confirm the effect of the induced sense and antisense constructs on the levels of the Emc expression (Emc has a predicted molecular weight of 22 kDa). As shown in Fig. 2A, Emc protein levels are highly elevated in heatshocked sense flies (lines *s1* and *s7b*) in comparison to the low endogenous Emc expression in wild-type *OrR* flies, whereas in antisense flies (line *as10c*) Emc protein is essentially undetectable (Fig. 2A) in comparison to beta-Tubulin. Similar results were obtained with immunohistochemistry with Emc antibody (Fig. 3A–C), as described below.

2.3. Specificity of the emc constructs

In order to establish if the *emc* sense and antisense constructs were generating phenotypes corresponding to those of known *emc* mutations we investigated their effects on bristle development in the thorax. Heatshock inducible lines *s1* (sense *emc*) and *as10c* (antisense *emc*) were used.

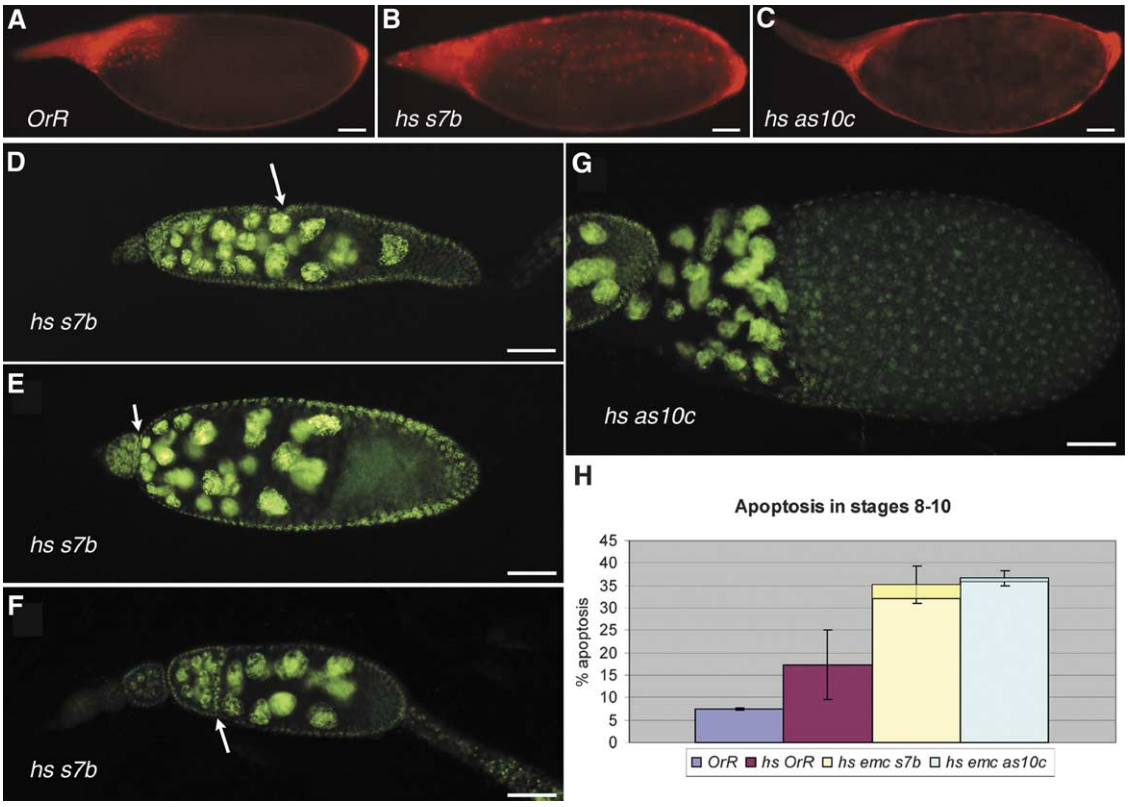

Fig. 3. (A–C) Immunohistochemistry with Emc antibody using stage 13 egg chambers. (A) Wild type Emc expression in the dorsal anterior using *OrR* ovaries. (B) Ectopic Emc in follicle cells laterally and posteriorly apart from the dorsal anterior in *hs s7b* sense *emc* flies. (C) Absence of Emc staining, even on the dorsal anterior in *emc* antisense flies *hs as 10c*. (D–G) Fused egg chambers from heatshocked sense and antisense *emc* fly ovaries. Nuclei are stained in green. (D) Arrow indicates a groove in the follicle cell layer where presumably this egg chamber should have been separated in two. (E–F) Arrows point to the irregular separation of two egg chambers. (G) A stage 11 fused egg chamber with more than fifteen nurse cells. The names of the fly lines used are written on each picture. (I) Graph showing the percentage of apoptotic egg chambers in wild-type and *emc* transgenic flies during stages 8–10.Vertical lines denote the standard deviation. Darker coloured boxes in the *s7b* and *as10c* columns represent the fraction of apoptotic egg chambers that also exhibit a fused phenotype. For heatshocked and non-heatshocked wild-type *OrR* flies this equals zero. Scale bars = 50 μm.

Females were allowed to lay eggs for 24 h, and then transferred to a new vial; this was repeated for 10 days to collect all developmental stages. The vials were then all heatshocked at once and the resulting flies were scored for bristle phenotypes.

Many flies had missing dorsocentral and scutellar bristles. However, the control wild type flies frequently had these phenotypes as well, when heatshocked. The heatshocked *emc* sense and antisense flies also showed phenotypes typical of *emc* mutations (Huang et al., 1995): extra dorsocentral and scutellar bristles, two bristles (scutellar and/or postalar) coming from one socket and short thick bristles in several locations. There were also flies with scutellar bristles facing in the wrong orientation. The frequency of occurrence of these abnormal and extra bristles is shown in Fig. 2B, and some of the phenotypes observed are shown in Fig. 2D–F, compared to the *OrR* phenotype (Fig. 2C).

Emc antibody staining in ovaries of wild type *OrR*, sense *s7b* and antisense *as10c* fly lines also confirms the specificity of the *emc* constructs and effectiveness of the heatshock regime. Emc is observed ectopically in the heatshocked sense line *s7b* (Fig. 3B—note the lateral/posterior follicle cells stained on the oocyte) compared to the wild type Emc expression pattern (Fig. 3A). At the same time there is absence of Emc staining from the heatshocked antisense line *as10c* (Fig. 3C) consistent with the 'knock-out' effect of the antisense construct.

2.4. Disruption and misexpression of emc affects egg chamber formation

In heatshocked sense and antisense *emc* ovaries we detected egg chambers fused to various extents (Fig. 3E–H). Sometimes there are 32 germline nuclei, presumably resulting from the fusion of two egg chambers, but usually there is incomplete fusion, resulting in one abnormally large egg chamber with an irregular number of nurse cell nuclei (e.g. 28 nurse cells—Fig. 3F) linked to an atrophic or underdeveloped egg chamber. These egg chambers were undergoing apoptosis but there were cases where development appeared to proceed to some extent (stage 11 egg chamber in Fig. 3H). This fused egg chamber phenotype was observed more in sense *emc* lines than in antisense *emc*

and wild-type flies never exhibited it, even when they were subjected to the same heatshock treatment as *emc* transgenic flies and the number of egg chambers undergoing apoptosis in their ovaries was increased (Fig. 3I).

2.5. Disruption and misexpression of emc affects dorsal follicle cell determination

The endogenous *emc* RNA expression analysis (Fig. 1C_{2-6}) and the enhancer trap line *261/31* (Fig. 1B_{1-4}) show that *emc* is expressed in the anterior follicle cells from stage 9–13. It is known that the dorsal–ventral axis is established by the EGFR signalling pathway at stage 9 and we postulated that *emc* may play a role in cell fate determination during this time. To investigate this we examined the effects of expressing both sense and antisense *emc* on eggshell patterning. Variation in expression of the *emc* sense and antisense due to position effects was expected, so an initial trial of 8 *emc* sense lines and 11 *emc* antisense lines was performed (standard heatchock, Fig. 5A,B), to establish which lines to use in a more detailed examination. The eggs were collected and examined for abnormal dorsal appendage formation, as this was likely to occur if there were defects in dorso–ventral patterning. Eggs were generally ventralised and their appendage phenotype ranged from appendages closer together or with fused bases to completely fused appendages. Occasionally they had wild-type looking appendages that were closer together at base (Fig. 4E); more frequently though they had wider dorsal appendages, usually shorter than wild-type (Fig. 4A) and irregular in shape (Fig. 4C–D,F–H), sometimes with split ends (Fig. 4G). Both sense and antisense heatshocked fly lines exhibited similar phenotypes.

We investigated the frequencies of phenotypes that arose following heatshock at different stages of oogenesis. To do this, females were heatshocked and the eggs collected at various time points after the heatshock. Fig. 5A shows variation in the frequency of abnormal phenotypes among the *emc* heatshocked sense fly lines, as expected. There are similarities between different insertion lines misexpressing the sense construct, with eggs collected 0–24 h after heatshock having the highest frequency of abnormal appendages. This shows that ectopic expression of *emc* in later stage egg chambers has the greatest effect upon dorsal patterning. The low percentages of abnormal egg chambers are to be expected, because not all egg chambers would have been at the appropriate point in dorsal–ventral axis determination to have an effect when the constructs were expressed during the short 45 min heatshock.

There is a greater variability in the frequency of eggs laid with abnormal appendages between different antisense lines disrupting *emc* (Fig. 5B), although the classes of abnormal phenotypes were similar to those of the sense lines. The heatshocked antisense *emc* lines laid abnormal eggs much later than the heatshocked sense *emc* lines, showing that disruption early in oogenesis has a greater phenotypical effect. Presumably this reflects the time needed for the already expressed *emc* RNA and protein to be turned over and disrupted by the antisense *emc* expression.

The heatshock method affects the numbers of abnormal eggs laid and the exact phenotypes observed. An alternative, more severe heatshock (see methods) led to similar phenotypes but eggs were frequently much smaller, irregular in shape and with fused appendages. Heatshock alone can cause abnormal dorsal appendage phenotypes (in *OrR*) and occasionally leads to the production of smaller eggs; however, the percentages are significantly lower than for *emc* mis-expression lines.

To investigate in detail the nature of the phenotypic changes in the appendages, eggs laid by females expressing *emc as10c* (standard heatshock regime) were collected and the spacing between the appendages was measured to see how much the appendages had moved. Since these were ventralised we compared them to wild type *OrR* eggs and to eggs laid by *gurken*^{22j} (*grk*^{22j}) females. The *grk*^{22j} flies have four copies of the wild type *grk* gene and the positioning on the appendages is influenced by *grk* via the EGFR pathway (Neuman-Silberberg and Schüpbach, 1994). Misexpression of *grk* causes a broader area of the egg to be dorsalised and the appendages are pushed further apart (Fig. 4B).

The frequency distribution (Fig. 5C) shows significant shifts in the spacing between the appendages in the eggs laid by *emc* antisense and *grk*^{22j} lines when compared to wild type eggs. The distance between appendages is bigger for the *grk*^{22j} line by approximately 17 μm. In heatshocked lines expressing *emc* antisense, the distance is smaller, i.e. the appendages are closer together, by approximately 15 μm. There is an interesting phenomenon showing that once the appendages get closer together than approximately 20 μm they fuse rather than form separately. To investigate this further and to investigate if the cells are different in number, shape or size we looked at the organisation of cells in the space between the appendages. Wild type eggs have elongated follicle cells between the dorsal appendages. The *emc* antisense mutants have a similar cellular shape, however, the cells between the *grk*^{22j} appendages are shorter and wider than in the *emc* antisense eggs and the wild type (data not shown). Thus, *emc* mutants do not change the shape of cells, but the number of cells between the appendages.

2.6. Analysis of emc homozygous mutant clones in follicle cells

Mosaic flies with *emc* homozygous clones were generated in order to investigate the effect of *emc* mutant clones in oogenesis and to confirm that the abnormal appendage phenotypes seen in the antisense analysis were due to perturbations in *emc* expression.

Follicle cell clones were observed in the ovaries of heatshocked *FLP*; *emc*^{1}, *FRT 80B/nlsGFP, FRT80B* flies as

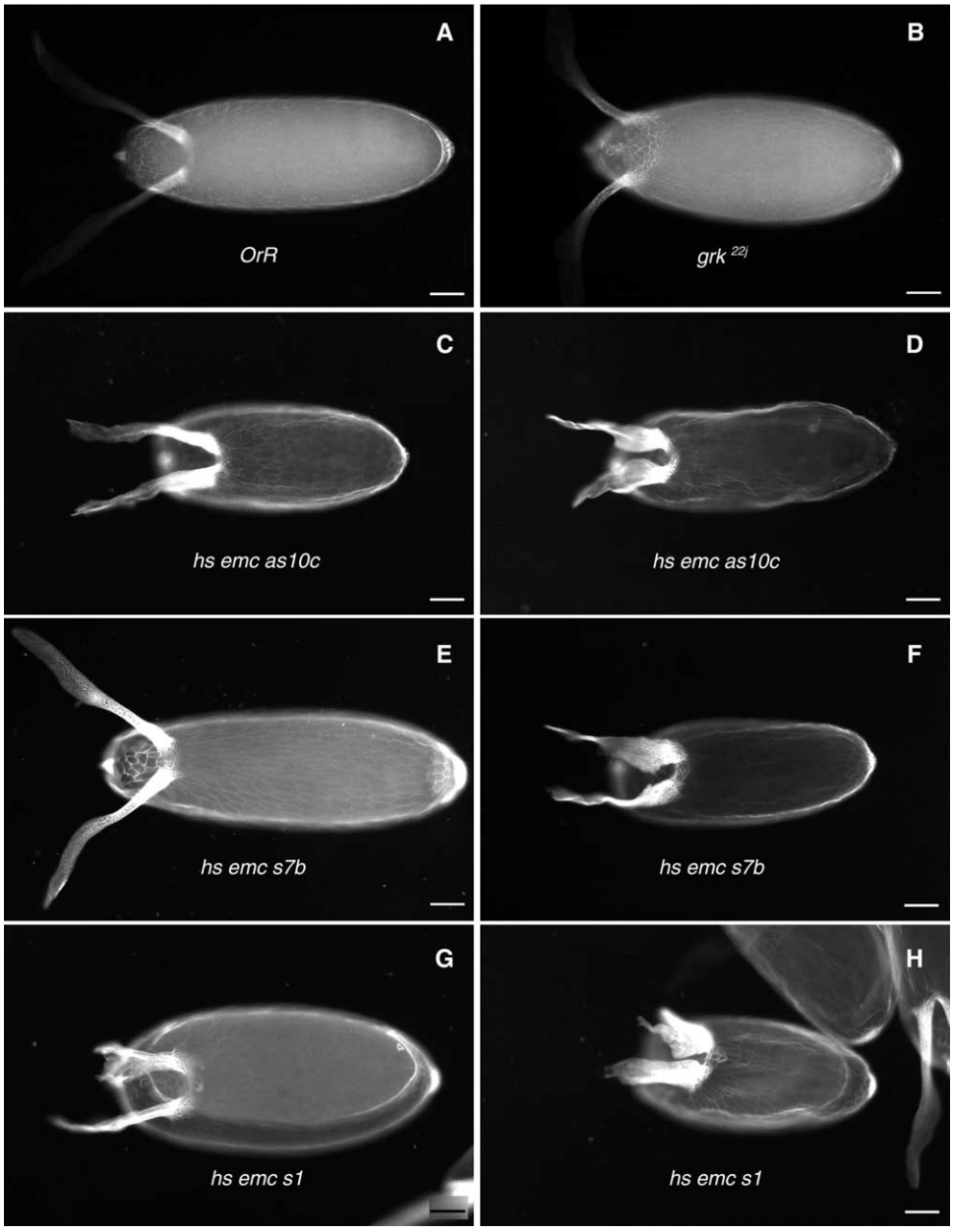

Fig. 4. The phenotypes observed in *OrR*, grk^{22j} and *emc* sense and antisense eggs. (A) *OrR*. (B) grk^{22j} with the dorsal appendages pushed further away from the dorsal midline. (C) and (D) Heatshocked *emc as10c* have appendages that are thicker and pushed closer to the dorsal midline. (E–H) are from two *emc* sense lines, *s7b* in (E) and (F) and *s1* in (G) and (H); they show the varying thickness and length of appendages and their abnormal tip morphology—these egg chambers are also ventralised. Note the wider and shorter cells between the appendages in (B). Scale bar = 50 μm. Transgenic eggs are generally smaller than wild-type.

shown in Fig. 6A,B. Occasionally the follicle cells within the clone would appear denser and smaller compared to their neighbouring follicle cells outside the clone (Fig. 6A) but the presence of *emc* mutant clones, even in quite large areas of the follicle cell monolayer (Fig. 6B) does not seem to have an immediately obvious morphological effect during oogenesis. However, the eggs laid by the heatshocked flies having *emc* clones in their ovaries have a high percentage of abnormal dorsal appendages. The most pronounced phenotype associated with the *emc* mutant clones is dorsal appendages with split ends ('antler'-type) to various degrees (Fig. 6D–H). Many other types of abnormally formed dorsal appendages were observed, ranging from complete absence of appendages with only some dorsal appendage material on the eggshell to appendages closer together or fused at base. Some of the phenotypes were also observed in eggs laid by heatshocked *OrR* flies but at a much lower percentage than the *emc* clones (Table 1); the 'antler'-type appendages though were unique to *emc* clones. Further evidence that these eggs came from egg chambers which had *emc* clones was the observation of abnormally large or misshapen cell imprints on the eggshell of the eggs with abnormal

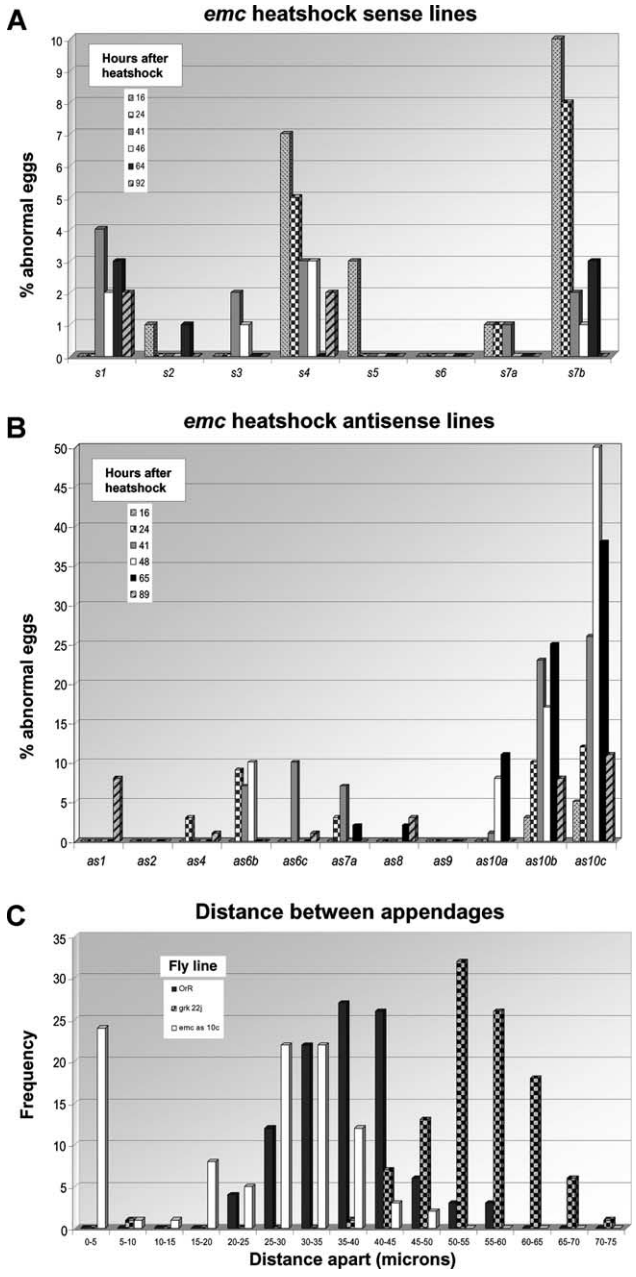

Fig. 5. (A) Graph representing the percentage of abnormal eggs laid from the 8 *emc* sense fly lines. Each line was heatshocked, separately, at 16 (spotted grey column), 24 (checked black/white), 41 (grey), 46 (white), 64 (black) and 92 (grey/black diagonal) hours before the eggs were collected for scoring and the percentage of abnormal eggs recorded. (B) Graph representing the percentage of abnormal eggs laid from the 11 *emc* antisense fly lines. Each line was heatshocked, separately, at 16 (spotted grey), 24 (checked black/white), 41 (grey), 48 (white), 65 (black) and 89 (grey/black upward diagonal column) hours before the eggs were collected for scoring and the percentage of abnormal eggs recorded. (C) Measurements of the distances between the dorsal appendages in *OrR* (black), grk^{22j} (checked grey/black) and *emc as10c* (white) (heatshocked 48 h prior to collecting for scoring) fly lines eggs. The distance between appendages is smaller in the *emc as10c* eggs compared to the wild-type as the appendages are much closer to the dorsal midline. The distance between appendages in the grk^{22j} fly line eggs is bigger compared to the wild-type as the appendages are further away from the dorsal midline. Statistical analysis of the results proved that the results are statistically significant at the 5% level.

appendages (Fig. 6I). This was never observed in heatshocked *OrR* flies, not even when they had abnormal dorsal appendages. Furthermore, the flies producing eggs with *emc* homozygous mutant clones also had extra (mostly dorsocentral) bristles in the notum (Fig. 6J–L), characteristic of *emc* mutations (Moscoso del Prado and Garcia-Bellido, 1984; Huang et al., 1995).

Antibody staining for Broad-Complex (Br-C) using *emc* mosaic flies shows that Br-C expression is not disrupted by the absence of *emc* from a clone of cells (Fig. 6M–O). This is true not only in stages 9–10 (Fig. 6M) when one could argue that Emc and Br-C do not co-localise (Fig. 7I), thus they would not be expected to be interacting, but also in stages 12 and 13 (Fig. 6N,O) where they would be expressed in the same subsets of dorsal anterior follicle cells.

2.7. *emc* and the Grk/EGFR signalling pathway

The dynamic late expression pattern of *emc* in oogenesis with the localisation in two dorsal anterior patches at stage 12 that correspond with the stage 12 expression pattern of Broad-Complex and the effect of *emc* misexpression, knock-out or clones on the egg dorsal appendage formation led us to suspect a link between *emc* and the Grk/EGFR pathway which is responsible for the establishment of the dorso–ventral (D–V) axis of the oocyte. We used in situ RNA hybridisation to investigate the position of *emc* with respect to genes that are well-known components of the Grk/EGFR (Grk/DER) signalling pathway.

As shown in Fig. 7C, in the ovaries of grk^{ED11} homozygous mutant flies *emc* ceases to be expressed in two distinct subsets of dorsal anterior follicle cells at stages 11 and 12 and is instead expressed in a broad dorsal anterior region covering the dorsal midline (compare with Fig. 7A). Mutant $gurken^{ED11}$ flies (Neuman-Silberberg and Schüpbach, 1993) lay ventralised eggs with the dorsal appendages being closer together and thinner than in the wild type (Fig. 7B); their phenotype ranges from a smaller distance between the appendages to appendages fused at the base (Fig. 7D), completely fused or a single very short appendage on the dorsal midline. *emc* mRNA is not expressed in the early stages of oogenesis and it is present in the centripetal cells at stages 9–10b (results not shown) but from stage 11 onwards its expression pattern changes to cover the dorsal midline in a single band in a manner reminiscent of the future appendage appearance of the laid egg. Therefore, we suggest that *emc* lies downstream of *gurken* in oogenesis.

Similarly, *emc* transcript expression pattern is disrupted by mutations in *fs(1)K10* and *Egfr* (*Torpedo*) genes (Fig. 7E–H′). Fs(1)K10 is required in the oocyte nucleus to restrict *grk* expression to the dorsal part of the anterior oocyte, most likely by interaction with Squid and Bruno proteins (Kelley, 1993; Norvell et al., 1999). Eggs mutant for $fs(1)K10^{4}$ are dorsalised, having two very wide lateral respiratory appendages (due to the—dorsal—region of *grk* expression expanding towards the ventral side) or an even

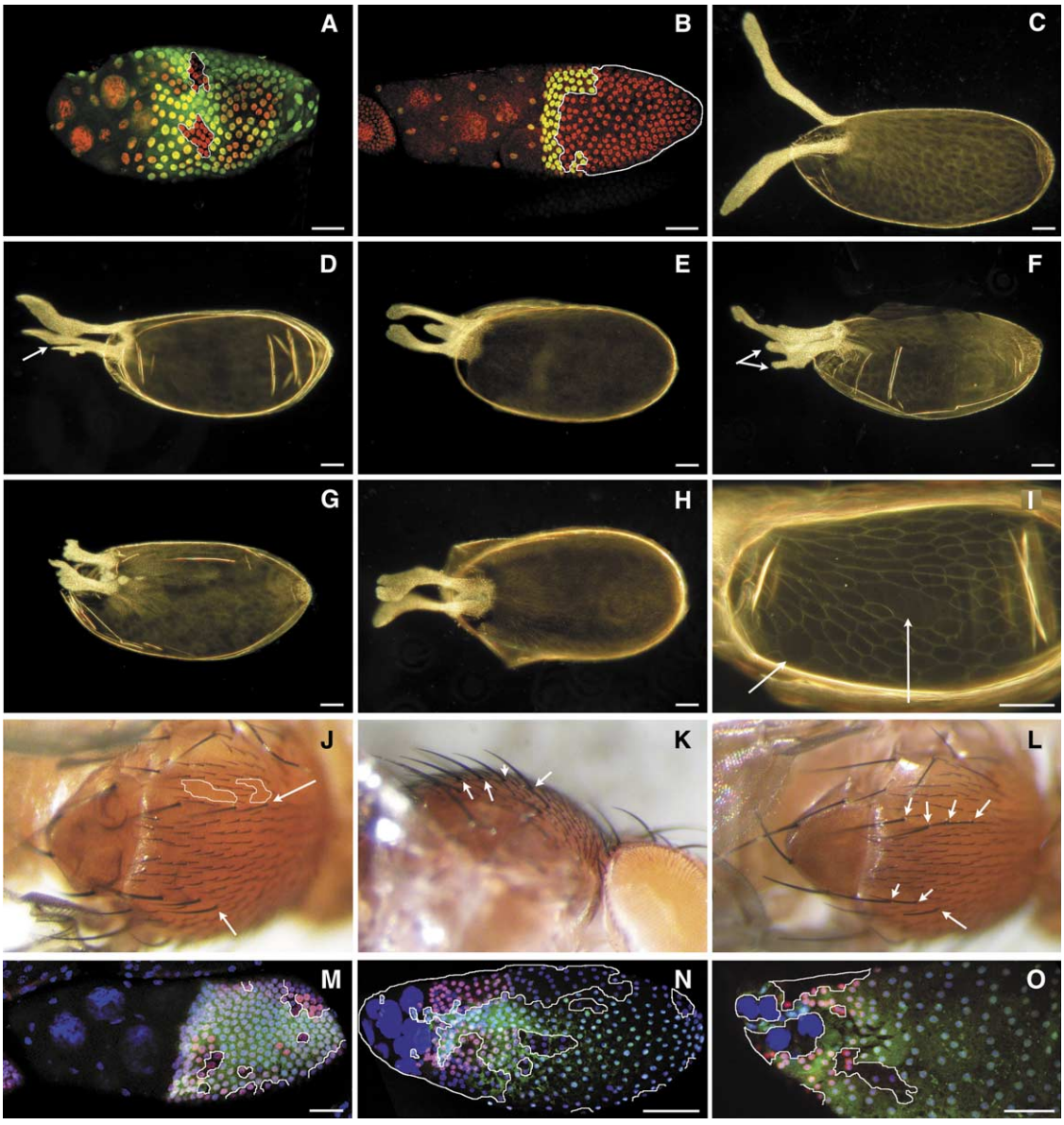

Fig. 6. Phenotypic effects of *emc* follicle cell clones. (A and B). Confocal micrographs of *emc* clones in ovaries. Nuclear GFP is green and all nuclei are in red (TO-PRO 3). Cells homozygous for emc^{1} do not express GFP and appear red rather than yellow in the overlays (clones circled with a white line). (C) Wild-type egg morphology. (D–H) Eggs from flies with *emc* follicle cell clones in ovaries. The dorsal appendages are closer together or fused at the base, have split ends (arrows), are thicker or occasionally shorter than wild type and sometimes have extra material. (I) Follicle cell imprints on the eggshell of a mosaic egg. Arrows point to the abnormally shaped large clones as opposed to the regular follicle cell pattern. (J–L) Mosaic flies have extra dorsocentral bristles (arrows) in the notum and the pattern of microchaetae can be disrupted in areas of *emc* clones (microchaetae absent from areas circled with a white line in (J)). (M–O). Confocal microscope overlays of *emc* clones (absence of green nuclear GFP staining), Broad-Complex antibody staining (red) and DNA staining (blue). The Broad-Complex expression pattern is not affected by the presence of *emc* clones; clones are circled with a white line. (M) Stage 10, (N) Stage 12 and (O) stage 13 egg chamber. Scale bars = 50 μm.

more extreme phenotype of a ring of dorsal appendage material all around the anterior of the egg (Fig. 7F,F′) because of *grk* transcripts being translated throughout the anterior of the oocyte. As shown in Fig. 7E *emc* transcripts are present in a ring all around the oocyte anterior at stage 12 instead of being restricted to two dorsal anterior follicle cell subsets, or, in milder phenotypes, *emc* is expressed in two lateral and a wide dorsal anterior follicle cell patch (Fig. 7E′). Top^{QY1} mutant flies (Schüpbach, 1987) laid ventralised eggs with a central, very thin and short dorsal appendage (Fig. 7H) or very little dorsal appendage material (Fig. 7H′). The expression of *emc* is changed accordingly, with *emc* mRNA being present in one dorsal, thinner and longer compared to the grk^{ED11} mutants, anterior group of follicle cells covering the dorsal midline at stage 12 (Fig. 7G); alternatively, *emc* is expressed in even smaller dorsal anterior spots or is absent from the follicle cells at stage 12 (Fig. 7G′), presumably reflecting the various

Table 1
Percentage of eggs with abnormal dorsal appendages from heatshocked wild-type *OrR* and mosaic *emc*[1] flies with follicle cell clones, at different times after heatshock

Collection time (h)	Eggs with abnormal dorsal appendages (%)	
	hs *OrR*	hs mosaic *emc*[1]
0–26	2.76	8.01*
26–38	9.75	13.93
38–52	0.0	2.68

Within the 0–26 h interval, the peak of eggs with abnormal DA laid from both fly lines was between 21 and 25 h after heatshock (data not shown). Statistical analysis (chi-square test) showed that the results are statistically significant at the 0.1% level for the 0–26 h after heatshock collection (chi-square = 19.41, $P < 0.001$), as indicated by the asterisk (*), but not for the 26–38 h and 38–52 h after heatshock collections (chi-square = 0.71 and 0.24, respectively; it should be $\geqq 3.84$ for significance at the 5% level). This means that for the last two samples the difference in the percentage of abnormality between mosaic and control flies may well be caused by the heatshock treatment but during the first 26 h after heatshock the abnormal appendage phenotypes in the mosaic flies are caused by the presence of follicle cell clones since the difference from the control flies is significant. The relatively low percentage of eggs with abnormal dorsal appendages can be explained as only a small proportion of egg chambers would be at the correct developmental stage during the heatshock. Furthermore, not all of the egg chambers would have *emc* homozygous mutant clones covering the specific subpopulations of follicle cells that affect appendage formation.

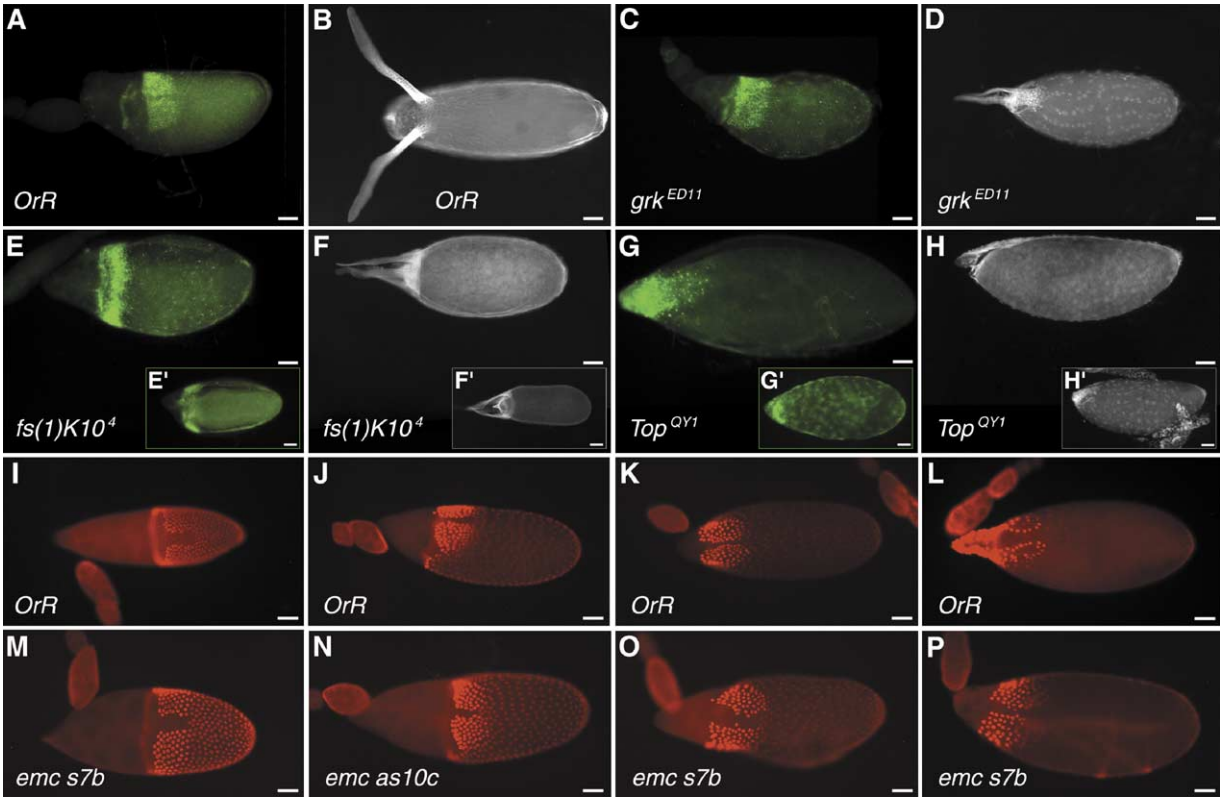

Fig. 7. (A–H′) In situ hybridisation with *emc* RNA on mutant backgrounds. (A, C, E, E′, G and G′) *emc* expression (green) in stage 12 egg chambers and (B, D, F, F′, H and H′) the corresponding dorsal appendage phenotype for each fly line. In (A) *emc* is expressed in two dorsal anterior subsets of follicle cells either side of a thin gap at the dorsal midline in wild-type *OrR* flies. *OrR* eggs have normal dorsal appendages (B). In grk^{ED11} mutant flies *emc* is expressed in a single broad dorsal anterior area covering the dorsal midline (C) and the eggs laid (D) have thin, shorter dorsal appendages fused at base (ventralised eggs) and are smaller than wild-type. *emc* is expressed in a ring of follicle cells all around the dorsal anterior (E) or in two lateral and a wide dorsal anterior patches (E′) in $fs(1)K10^4$ mutants, resulting in dorsalised eggs shorter and more round than wild-type, with four appendages all around the anterior (F) or two wide lateral appendages with appendage material connecting them (F′). In Top^{QY1} mutants *emc* is expressed in a single patch over the dorsal midline (G) or is absent from follicle cells (G′). The corresponding eggs (ventralised) have either one very short, thin dorsal appendage (H) or very little chorionic appendage material at the anteriormost region of the egg (H′). (I–P) Immunohistochemistry with a Br-C antibody. (I–L) show the expression pattern of Broad-Complex in *OrR* flies at stages 10 (I), early (J) and late (K) stage12 and stage 13 (L). (M–P) show Br-C in *emc* sense and antisense fly lines. Normal Br-C expression is not disrupted in mid-oogenesis in the sense line *s7b* (M), but later at stage 12 the shape of the patches of follicle cells expressing Br-C is affected (O and P). Disruption of normal Emc expression in the antisense line *emc as10c* does not inhibit Br-C expression and disrupts the shape of the Br-C - expressing patches only mildly (N). Scale bars = 50 μm.

degrees of ventralisation of the egg caused by the absence of EGFR signalling. These results suggest that *emc* expression is involved in the commitment to dorsal appendage fate of the follicle cells in which it is expressed, and that it is downstream of the *fs(1)K10* and *Egfr/Top* genes in the Grk/EGFR signalling pathway.

2.8. Broad-Complex expression pattern is disrupted in emc sense and, to a lower extent, antisense transgenic flies

Since the *emc* late expression pattern in oogenesis is reflected in the dorsal appendage phenotype observed, we used an antibody against the Broad-Complex (Br-C) core domain (Tzolovsky et al., 1999) on *emc hs as10c* and *s7b* fly lines. The Br-C locus is required for dorsal appendage morphogenesis and is an effector of the Grk/EGFR pathway on D–V axis formation while Dpp signalling is necessary for its correct expression along the A–P axis (Deng and Bownes, 1997). Br-C expression pattern is quite different from that of *emc* in early and mid-oogenesis (Fig. 7I) up to stage 12 where Br-C is expressed in two subsets of dorsal anterior follicle cells either side of the dorsal midline exactly like *emc*. The same pattern is followed by Br-C and Emc proteins at stage 12 and 13 (Fig. 7J–L). In the sense *emc* line *s7b* the late Br-C protein expression pattern is disrupted more often than in the antisense *emc* line *as10c* (Fig. 7O,P, compared with Fig. 7J,K), suggesting that *emc* affects *Broad-Complex* expression with respect to the dorsal appendage-producing follicle cells. Its function must be one of refining the dorsal appendage position rather than determining whether there will be dorsal appendages produced or not, since in *emc* antisense heatshocked flies BR-C was still expressed, even though Emc was knocked-out, and the BR-C expression pattern was usually normal (Fig. 7N); Br-C was also not affected in Emc null follicle cell clones (Fig. 6N,O). In the sense *emc* flies, where *emc* was ectopically expressed, Br-C was likely to be expressed in extra follicle cells, suggesting that *emc* needs to be correctly localised for the correct localisation of Br-C in the follicle cells producing the dorsal appendages, but that there must be other genes responsible for regulating the expression itself of BR-C acting on those subsets of follicle cells. Expression of Br-C during early and mid- oogenesis was not affected by *emc* misexpression (Fig. 7M compared to Fig. 7I).

3. Discussion

emc appears to have a key role in cell fate determination in somatic cells during the process where follicle cells surround the oocyte and in dorsal appendage positioning.

We have shown using β-galactosidase staining of an enhancer-trap line, in situ hybridisation to mRNA and antibody staining that *emc* has a dynamic expression pattern in oogenesis. Expression is observed both in the germ cells and in the somatic follicle cells. Maternal Emc is already known to be essential for sex determination in embryogenesis (Younger-Shepherd et al., 1992) thus the expression observed in the nurse cell nuclei was expected.

Ectopic *emc* overexpression using heatshock sense fly lines leads to a large number of fused egg chambers that eventually undergo apoptosis. Most of the abnormal egg chambers seem to be fused longitudinally, as they appear to be very long and thin. A different type of fusion is present using antisense *emc* knock-out: fusion seems to happen laterally since egg chambers that can proceed up to stage 11 of oogenesis with a normally developing oocyte and double the number of nurse cells were observed. This phenotype was not observed in heatshocked *OrR* flies. These phenotypes suggest abnormalities during enveloping of the germline cysts in germarium region 2, when egg chambers become assembled and are characteristic of mutants for *Notch*, *Delta*, *daughterless* and of ectopic *hedgehog* expression (Goode et al., 1996; Forbes et al., 1996). In this case *emc* would be implicated in enveloping the germ cell cluster, consistent with presence of the protein in the germarium and with recent findings by Adam and Montell (2004) who observe similar fused egg chambers following overexpression of *emc*. They confirm the relationship of *emc* with the Notch signalling pathway in oogenesis by identifying *emc* as an effector of Notch signalling in the differentiation of follicle cells and suggest that the fused phenotypes are caused by the failure of polar cells to differentiate (polar cells are required to induce stalks which separate developing egg chambers in the ovariole) (Adam and Montell, 2004).

Emc also functions later in development in the follicle cells, during the regulation of dorsal appendage formation, a procedure controlled by the Grk/EGFR pathway. The chorionic appendages are moved closer together and often fuse when *emc* levels are reduced by antisense expression. Similar results are seen when *emc* is removed in clones of cells, where the appendages are frequently split at their ends. We have demonstrated that *emc* mRNA expression is affected by the disruption of the normal dorso–ventral axis formation caused by mutants for *fs(1)K10*, *gurken* and *Egfr*. Specifically, the *emc* expression pattern follows the ventralisation caused by grk^{ED11} and Top^{QY1} and the dorsalisation caused by $fs(1)K10^{4}$. Therefore *emc* functions downstream of *fs(1)K10*, *gurken* and *Egfr* and upstream of the finally formed dorsal appendages.

Further support for the role of *emc* in dorsal appendage morphogenesis comes from our finding that *emc* ectopic expression affects the expression pattern of Broad-Complex, a transcription factor that regulates dorsal appendage formation (Deng and Bownes, 1997). In *emc* sense transgenic lines Br-C protein is expressed in a more irregular pattern than normal, mainly at stages 12–13. Br-C protein expression pattern coincides with that of *emc* mRNA at stage 12 of oogenesis as they are both expressed in two dorsal anterior subsets of concentrated follicle cells

either side of the dorsal midline (these 'patches' of follicle cells are discrete and visible also with nuclear markers) and in late stage 12–13 they follow the same pattern of being expressed in the migrating follicle cells that are forming the dorsal chorionic appendages. In *emc* sense flies the borders of Br-C expression are irregular and there are follicle cells in the gap between the two patches or around their borders that express Br-C. So Br-C must lie downstream of *emc* since its expression is affected by *emc* misexpression. However, *emc* is not the gene that activates Br-C since Br-C protein is expressed in the normal wild-type pattern in *emc* homozygous mutant follicle cell clones and in *emc* antisense lines where *emc* is knocked-out (or there are minor disruptions in the Br-C expression area in antisense compared with sense lines); rather, it seems that *emc* must be necessary for modulating Br-C within the areas of *emc* expression.

It would be interesting to investigate how the role of *emc* acting downstream of *Notch* (Adam and Montell, 2004) may be interlinked with its interaction with the Grk/EGFR pathway we report here. In other tissues such as the developing eye and wing *emc* is required for a number of processes and acts in parallel or is regulated by many different signals, including the EGFR and the Notch signalling pathways (de Celis, 1998; Baonza and Freeman, 2001). Further investigation of the multiple roles of *emc* in oogenesis and their specific timing should help to unravel the complexities of producing a viable, morphologically normal, egg.

4. Experimental procedures

4.1. Drosophila stocks

Wild type stocks: *Oregon R* (*OrR*) and w^k (Lüning, 1981). LacZ line, *261/31* (Deak et al., 1997). Transgenic *emc* lines were generated in our lab. Mutant alleles used: grk^{22j} (Neuman-Silberberg and Schüpbach, 1994); grk^{ED11} (Neuman-Silberberg and Schüpbach, 1993), top^{QY1} (Schüpbach, 1987). All other stocks were obtained from the Bloomington *Drosophila* Stock Center, Indiana University, USA.

4.2. In situ hybridisation in ovaries with DIG-labelled DNA probe

The protocol is based on a procedure previously described (Tautz and Pfeifle, 1989) with minor modifications.

4.3. Fluorescent in situ hybridisation in ovaries with DIG-labelled RNA probe

The protocol is based on the Tautz and Pfeifle (1989) procedure with the following modifications. Ovaries were collected and fixed in PMS, washed for 3×10 min in PBT, equilibrated for 10 min in RNA Hybrix/PBT (1:1) and then prehybridised for 1 h at 65 °C in RNA Hybrix (50% deionised formamide, 5× SSC, 100 μg/ml tRNA (RNase free), 50 μg/ml Heparin, 0.1% Tween 20). The ovaries were hybridised overnight at 65 °C in RNA Hybrix containing digoxigenin-labelled RNA probe. Templates were transcribed with either T7 or T3 polymerases (Ambion) using DIG RNA Labelling Mix (Roche, #1 277 073), to generate antisense and sense probes, the latter being used as a negative control. Detection was carried out with 1:80 dilution of anti-DIG HRP antibody (DakoCytomation, #D5101) for 1 h at room temperature (RT). For visualisation TSA-Cy3 reagent (PerkinElmer, #SAT704A) was used according to manufacturer's instructions. Ovaries were mounted in Vectashield (Vector Laboratories, #H1000) for observation.

4.4. Immunohistochemistry and western analysis

Ovaries were dissected in Ringer's and fixed for 20 min in PMS, washed with PBTW (PBS containing 0.5% Triton X-100) and incubated with 5% NGS (Normal Goat Serum) in PBTW for 1 h. To stain for Broad Complex (Br-C), a mouse anti-Core antibody (gift from G. Guild, University of Pennsylvania), which can recognise all Br-C isoforms was used in 1:60 dilution. An Emc rabbit antibody (provided by L.Y. Jan and Y.N. Jan, University of California, San Francisco) was used in 1:600 dilution. Secondary antibodies were either Alexa 488 or Alexa 568-conjugated (Molecular Probes, Invitrogen) and used in 1:400 dilution.

For the purposes of immunoblotting proteins extracted from *s1, s7b, as10c* and *OrR* ovaries were separated by SDS-PAGE electrophoresis and transferred onto reinforced nitrocellulose membrane (Schleicher and Schuell, Optitran BA-S83). To detect Emc we used the Emc antibody in 1:6000 dilution and then an anti-rabbit HRP-conjugated secondary antibody (Sigma, #A8275) in 1:4000 dilution. To visualise the immunoreactive bands the membrane was incubated for 1 min in chemiluminescent solution made up of 1.25 mM Luminol, 0.2 mM *p*-Coumaric acid and 100 mM Tris–HCl pH 8.5. Quantification of band intensity was done with ImageJ software (http://rsb.info.nih.gov/ij/).

4.5. Nuclear staining

For multicolour imaging DNA was counterstained with the appropriate nuclear stain. For blue colour DNA was stained with Hoechst 33258 (1 μg/ml) for 5 min in PBT. Nuclear yellow (Sigma, #N2137) was also used in 1 μg/ml concentration. For green colour we used Sytox Green (Molecular Probes, Invitrogen) at 150 nM concentration whereas TO-PRO-3 (Molecular Probes, Invitrogen), which can be detected in the infrared channel, was used at 250 nM concentration. No RNase digest was necessary when staining with Hoechst or Nuclear Yellow. Sytox Green

and TO-PRO-3 can stain DNA as well as RNA, therefore samples were pre-digested in 250 μg/ml RNase-A for 5–10 min. Stained samples were examined and captured digitally either on a Zeiss Axiophot fluorescent microscope or a Leica TCS SP confocal microscope.

4.6. Plasmid rescue

Genomic DNA from the *P{lacW}* enhancer-trap line *261/31* was digested with *Eco*RI and subsequently ligated overnight at 4 °C. The ligated DNA was used to transform XL1 Blue competent cells. Ampicillin selection allowed the isolation of colonies carrying the Amp^R part of *P{lacW}* plus any additional flanking genomic DNA. Plasmid DNA from two of these colonies was digested with *Eco*RI and *Bam*HI. The resulting fragment (the DNAs from both colonies were identical) was subcloned in pBluescript (SK-). M13–20 and Reverse primers were used to sequence the flanking genomic DNA and determine the P-element insertion site.

4.7. β-galactosidase staining

Female flies fed with yeast for 3 days were dissected in PBS. Ovaries were dissected and fixed for 5 min in 4% *p*-Formaldehyde in PBT, then stained overnight in 100 μl Staining Solution (10 mM Phosphate buffer, pH 7.0, 8 mM $K_3[Fe(CN)_6]$, 8 mM $K_4[Fe(CN)_6]$, 150 mM NaCl and 1 mM $MgCl_2$) to which 2.5 μl 8% X-Gal (5-Bromo-4-chloro-3-indolyl-β-D-galactoside) were added. The stained ovaries were rinsed in PBS and mounted in PBS/Glycerol (1:4).

4.8. Production of emc antisense and sense lines

To generate transgenic *emc* sense and antisense flies, a full length *emc* cDNA was used (Garrell and Modolell, 1990). *emc* cDNA was cut with a pair of restriction enzymes, *Eco*RI and *Bam*HI or *Spe*I and *Hinc*II. The resulting *Eco*RI–*Bam*HI and *Spe*I–*Hinc*II fragments of *emc* cDNA were ligated into *pCaSpeR-hs* cut with either *Stu*I and *Bgl*I or *Xba* and *Hpa*I, forming the *pHS-emc* (sense) and *pHS-as-emc* (anti-sense) constructs, respectively. Both constructs were then introduced into w^{1118} flies by P element mediated germline transformation (Spradling, 1986).

4.9. Heatshock regimes for expression of sense and antisense constructs

To investigate the bristles on the thorax batches of flies were allowed to lay eggs in vials for 24 h and then transferred to new vials. The vials were allowed to age for 0–11 days prior to heatshock at 39 °C for 45 min followed by 30 min at 25 °C and a further 45 min at 39 °C. The vials were then retained until adults hatched and the bristle patterns were observed. For most of the studies on dorsal appendage morphogenesis we used small cages, so that the eggs laid could be collected on agar plates. The heatshock regime was the same as above, except that in this case the adults were heatshocked prior to collecting eggs or observing ovaries. In an alternative, more severe, regime flies were heatshocked for 30 min in vials placed in 37 °C water bath. Heatshocked flies were transferred back to 25 °C. Eggs were subsequently collected from 0 to 48 h after heatshock.

For the mutant background experiments flies were heatshocked by transferring to vials placed in a 37 °C water bath for 1 h.

4.10. Induction of mitotic recombination by FLP/FRT system

For the generation of $emc^{1-/-}$ somatic follicle cell clones, ♂ y^1 w^{1118} $P\{ry^{+t7.2}=70FLP\}3F/Dp(1;Y)y^+$; *TM2/TM6C,* Sb^1 were crossed with ♀ w^{1118}; $P\{w^{+mC}=Ubi-GFP(S65T)nls\}3L$ $P\{ry^{+t7.2}=neoFRT\}80B/TM3$, Sb^1, female progeny of the genotype y^1 w^{1118} $P\{ry^{+t7.2}=70FLP\}3F/w^{1118}$; $P\{w^{+mC}=Ubi-GFP(S65T)nls\}3L$ $P\{ry^{+t7.2}=neoFRT\}80B/TM6C$, Sb^1 were selected and crossed with ♂ *w**; $emc^1P\{ry^{+t7.2}=neoFRT\}80B/TM6B$, Tb^1 (Xu and Rubin, 1993). Male progeny carrying the *FLP*;emc^1/*Ubi−GFP(S65T)nls* genes were selected and crossed with *w**; $emc^1P\{ry^{+t7.2}=neoFRT\}80B/TM6B$, Tb^1 female flies. Non-Tb 3rd instar larvae were selected and allowed to hatch. Female flies of the genotype y^1 w^{1118} $P\{ry^{+t7.2}=70FLP\}3F/w*$; emc^1 $P\{ry^{+t7.2}=neoFRT\}80B/P\{w^{+mC}=Ubi-GFP(S65T)nls\}3L$ $P\{ry^{+t7.2}=neoFRT\}80B$ were heatshocked for 1 h at 39 °C and then transferred to 25 °C in small cages. Embryos were collected in grape juice agar plates (per 400 ml: 9 g agar, 10 g sucrose, 100 ml grape juice, 1 ml 10% Nipagin (in 95% EtOH) in dH_2O) every 6–24 h and scored for abnormal appendage formation. Ovaries were dissected from heatshocked flies at 10 h intervals after heatshock. Alternatively, flies were yeasted for 3 days at 25 °C, then heatshocked for 1 h at 37 °C in a water bath, transferred to 25 °C for 4 h, heatshocked again at 37 °C for 1 h and transferred to 25 °C in yeasted vials. Ovaries were dissected 3–5 days after heatshock.

Acknowledgements

We thank L.Y. Jan and Y.N. Jan for the *emc* antibody. This project was supported by a Wellcome Trust grant (DZ and DC), a BBSRC studentship to KL, a Darwin Trust studentship to GT and SP and a Nuffield undergraduate summer studentship to EA (now studying for a PhD at Cambridge University). PT and GK were honours students; PT is now studying for a PhD at Edinburgh University. All work was undertaken at the Institute of Cell and Molecular Biology, University of Edinburgh.

References

Adam, J.C., Montell, D.J., 2004. A role for extra macrochaetae downstream of Notch in follicle cell differentiation. Development 131, 5971–5980.

Baonza, A., Freeman, M., 2001. Notch signalling and the initiation of neural development in the *Drosophila* eye. Development 128, 3889–3898.

Baonza, A., de Celis, J.F., Garcia-Bellido, A., 2000. Relationships between *extramacrochaetae* and Notch signalling in *Drosophila* wing development. Development 127, 2383–2393.

Benezra, R., Davis, R.L., Lockshon, D., Turner, D.L., Weintraub, H., 1990. The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. Cell 61, 49–59.

Botas, J., Moscoso del Prado, J., Garcia-Bellido, A., 1982. Gene-dose titration analysis in the search of trans-regulatory genes in *Drosophila*. Eur. Mol. Biol. Org. J. 1, 307–310.

Cabrera, C.V., Alonso, M.C., Huikeshoven, H., 1994. Regulation of scute function by *extramacrochaete* in vitro and in vivo. Development 120, 3595–3603.

Campuzano, S., 2001. Emc, a negative HLH regulator with multiple functions in *Drosophila* development. Oncogene 20, 8299–8307.

Cooperstock, R.L., Lipshitz, H.D., 2001. RNA localization and translational regulation during axis specification in the *Drosophila* oocyte. Int. Rev. Cytol. 203, 541–566.

Cubas, P., Modolell, J., Ruiz-Gomez, M., 1994. The helix-loop-helix extramacrochaetae protein is required for proper specification of many cell types in the *Drosophila* embryo. Development 120, 2555–2566.

Davis, R.L., Cheng, P.F., Lassar, A.B., Weintraub, H., 1990. The MyoD DNA binding domain contains a recognition code for muscle-specific gene activation. Cell 60, 733–746.

Deak, P., Omar, M.M., Saunders, R.D., Pal, M., Komonyi, O., Szidonya, J., Maroy, P., Zhang, Y., Ashburner, M., Benos, P., Savakis, C., Siden-Kiamos, I., Louis, C., Bolshakov, V.N., Kafatos, F.C., Madueno, E., Modolell, J., Glover, D.M., 1997. P-element insertion alleles of essential genes on the third chromosome of *Drosophila melanogaster* : correlation of physical and cytogenetic maps in chromosomal region 86E–87F. Genetics 147, 1697–1722.

de Celis, J.F., 1998. Positioning and differentiation of veins in the *Drosophila* wing. Int. J. Dev. Biol. 42, 335–343.

Deng, W.M., Bownes, M., 1997. Two signalling pathways specify localised expression of the Broad-Complex in *Drosophila* eggshell patterning and morphogenesis. Development 124, 4639–4647.

Deng, W.M., Bownes, M., 1998. Patterning and morphogenesis of the follicle cell epithelium during *Drosophila* oogenesis. Int. J. Dev. Biol. 42, 541–552.

Deng, W., Leaper, K., Bownes, M., 1999. A targeted gene silencing technique shows that *Drosophila myosin* VI is required for egg chamber and imaginal disc morphogenesis. J. Cell. Sci. 112 (Pt 21), 3677–3690.

Ellis, H.M., 1994. Embryonic expression and function of the *Drosophila* helix-loop-helix gene, extramacrochaetae. Mech. Dev. 47, 65–72.

Ellis, H.M., Spann, D.R., Posakony, J.W., 1990. extramacrochaetae, a negative regulator of sensory organ development in *Drosophila*, defines a new class of helix-loop-helix proteins. Cell 61, 27–38.

Forbes, A.J., Lin, H., Ingham, P.W., Spradling, A.C., 1996. *hedgehog* is required for the proliferation and specification of ovarian somatic cells prior to egg chamber formation in *Drosophila*. Development 122, 1125–1135.

Garrell, J., Modolell, J., 1990. The *Drosophila extramacrochaetae* locus, an antagonist of proneural genes that, like these genes, encodes a helix-loop-helix protein. Cell 61, 39–48.

Goode, S., Melnick, M., Chou, T.B., Perrimon, N., 1996. The neurogenic genes egghead and brainiac define a novel signaling pathway essential for epithelial morphogenesis during *Drosophila* oogenesis. Development 122, 3863–3879.

Huang, F., van Helden, J., Dambly-Chaudiere, C., Ghysen, A., 1995. Contribution of the gene extramacrochaete to the precise positioning of bristles in *Drosophila*. Roux's Arch. Dev. Biol. 204, 336–343.

Kelley, R.L., 1993. Initial organization of the *Drosophila* dorsoventral axis depends on an RNA-binding protein encoded by the *squid* gene. Genes Dev. 7, 948–960.

King, R.C., 1970. Ovarian Development in *Drosophila melanogaster*. Academic Press, New York.

Lüning, K.G., 1981. Genetics of inbred *Drosophila melanogaste*. Induction of marker genes and preliminary recombination tests. Hereditas 95, 181–188.

Massari, M.E., Murre, C., 2000. Helix-loop-helix proteins: regulators of transcription in eukaryotic organisms. Mol. Cell. Biol. 20, 429–440.

Morimoto, A.M., Jordan, K.C., Tietze, K., Britton, J.S., O'Neill, E.M., Ruohola-Baker, H., 1996. *Pointed*, an ETS domain transcription factor, negatively regulates the EGF receptor pathway in *Drosophila* oogenesis. Development 122, 3745–3754.

Moscoso del Prado, J., Garcia.-Bellido, A., 1984. Genetic regulation of the achaete-scute complex of *Drosophila melanogaster*. Roux's Arch. Dev. Biol. 193, 242–245.

Neuman-Silberberg, F.S., Schupbach, T., 1993. The *Drosophila* dorsoventral patterning gene gurken produces a dorsally localized RNA and encodes a TGF alpha-like protein. Cell 75, 165–174.

Neuman-Silberberg, F.S., Schupbach, T., 1994. Dorsoventral axis formation in *Drosophila* depends on the correct dosage of the gene gurken. Development 120, 2457–2463.

Norvell, A., Kelley, R.L., Wehr, K., Schupbach, T., 1999. Specific isoforms of squid, a *Drosophila* hnRNP, perform distinct roles in Gurken localization during oogenesis. Genes Dev. 13, 864–876.

Queenan, A.M., Ghabrial, A., Schupbach, T., 1997. Ectopic activation of torpedo/Egfr, a *Drosophila* receptor tyrosine kinase, dorsalizes both the eggshell and the embryo. Development 124, 3871–3880.

Ruohola-Baker, H., Grell, E., Chou, T.B., Baker, D., Jan, L.Y., Jan, Y.N., 1993. Spatially localized *rhomboid* is required for establishment of the dorsal–ventral axis in *Drosophila* oogenesis. Cell 73, 953–965.

Schupbach, T., 1987. Germ line and soma cooperate during oogenesis to establish the dorsoventral pattern of egg shell and embryo in *Drosophila melanogaster*. Cell 49, 699–707.

Spradling, A.C., 1986. P element-mediated transformation. In: Robert, D.B. (Ed.), *Drosophila*: A Practical Approach. IRL Press, Oxford, England, pp. 175–197.

Spradling, A.C., 1993. Developmental genetics of oogenesis. In: Bate, M., Martinez-Arias, A. (Eds.), The Development of *Drosophila melanogaster*. Cold Spring Harbor Laboratory Press, New York, pp. 1–70.

Tautz, D., Pfeifle, C., 1989. A non-radioactive in situ hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene hunchback. Chromosoma 98, 81–85.

Tzolovsky, G., Deng, W.M., Schlitt, T., Bownes, M., 1999. The function of the broad-complex during *Drosophila melanogaster* oogenesis. Genetics 153, 1371–1383.

van Buskirk, C., Schupbach, T., 1999. Versatility in signalling: multiple responses to EGF receptor activation during *Drosophila* oogenesis. Trends. Cell. Biol. 9, 1–4.

van Doren, M., Ellis, H.M., Posakony, J.W., 1991. The *Drosophila* extramacrochaetae protein antagonizes sequence-specific DNA binding by daughterless/achaete-scute protein complexes. Development 113, 245–255.

Wasserman, J.D., Freeman, M., 1998. An autoregulatory cascade of EGF receptor signaling patterns the *Drosophila* egg. Cell 95, 355–364.

Xu, T., Rubin, G.M., 1993. Analysis of genetic mosaics in developing and adult *Drosophila* tissues. Development 117, 1223–1237.

Younger-Shepherd, S., Vaessin, H., Bier, E., Jan, L.Y., Jan, Y.N., 1992. deadpan, an essential pan-neural gene encoding an HLH protein, acts as a denominator in *Drosophila* sex determination. Cell 70, 911–922.

Zhao, D., Bownes, M., 1999. Misexpression of argos, an inhibitor of EGFR signaling in oogenesis, leads to the production of bicephalic, ventralized, and lateralized *Drosophila* melanogaster eggs. Dev Genet. 25, 375–386.